Penetration of *Salmonella enteritidis* and *Salmonella heidelberg* into Egg Yolks in an In Vitro Contamination Model

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ABSTRACT Eggs that harbor *Salmonella* in their edible contents pose a significant risk of transmitting disease to consumers. Although *Salmonella* deposition inside yolks does not usually occur at a high frequency in naturally contaminated eggs, bacterial penetration through the vitelline membrane could lead to rapid and extensive multiplication in the nutrient-rich yolk contents. The present study used an in vitro egg contamination model to assess the ability of *Salmonella* strains to penetrate the vitelline membrane and multiply inside yolks. An *S. enteritidis* strain and 2 *Salmonella heidelberg* strains, initially inoculated onto the outside of the vitelline membrane, were able to enter the yolk contents (at frequencies ranging from 10 to 25% of experimentally contaminated eggs) during 24 h of incubation at 30°C. Variants of these parent strains, obtained by in vivo passage into eggs laid by infected hens, penetrated the yolk membrane at significantly higher frequencies. These results demonstrate that pathogens such as *S. enteritidis* and *S. heidelberg* can penetrate into and begin to multiply inside the yolks of contaminated eggs during the first day of storage at warm temperatures.

(*Key words*: *Salmonella enteritidis*, *Salmonella heidelberg*, yolk, vitelline membrane, penetration)

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INTRODUCTION

The importance of contaminated eggs in the transmission of *Salmonella enterica* serovar Enteritidis (*S. enteritidis*) to humans has been recognized for nearly 2 decades throughout the world (Angulo and Swerdlow, 1999; Centers for Disease Control and Prevention, 2003). Both naturally and experimentally infected chickens have been observed to produce eggs containing *S. enteritidis* in their liquid interior contents (Humphrey et al., 1989, 1991; Gast and Beard, 1990; Gast and Holt, 2000b;). Substantial public and private resources have been invested in control programs to reduce the occurrence of *S. enteritidis* infections in laying flocks and to improve storage and preparation practices for eggs and egg-containing foods (Hogue et al., 1998; President's Council on Food Safety, 1999). The Centers for Disease Control and Prevention have subsequently implicated *S. enterica* serovar Heidelberg (*S. heidelberg*) as a potentially significant source of egg-associated human disease (Chittick et al., 2004; Hennessy et al., 2004). A recent experimental infection study demonstrated that some *S. heidelberg* isolates can invade reproductive tissues of laying hens and are thereby deposited inside developing eggs in a manner similar to *S. enteritidis* (Gast et al., 2004).

Refrigeration of eggs is often identified as one of the most critical issues in minimizing the risks associated with *Salmonella* contamination of eggs (President's Council on Food Safety, 1999; U. S. Department of Agriculture, 1998). Because freshly laid eggs are very rarely reported to harbor more than a few hundred *S. enteritidis* cells (Humphrey et al., 1989, 1991; Gast and Beard, 1992; Gast and Holt, 2000b; Chen et al., 2002), prompt refrigeration to growth-inhibiting internal temperatures (7.2°C or lower) can reduce the probability that consumers will be exposed to doses of the pathogen sufficient to cause disease. However, the prospective protective efficacy of refrigeration can be heavily influenced by the location of *Salmonella* contaminants within eggs. Although *Salmonella* can persist or even grow slowly in albumen (Baron et al., 1997; Gast and Holt, 2000a; Cogan et al., 2001; Messens et al., 2004), egg yolk supports rapid bacterial multiplication (Clay and Board, 1991; Humphrey and Whitehead, 1993; Braun and Fehlhaber, 1995; Gast and Holt, 2000a). Accordingly, the degree of urgency for rapidly attaining growth-inhibiting internal temperatures inside eggs is directly related to the likelihood that the pathogen is present inside the yolk. Infected hens have been reported to deposit *S. enteritidis* in either yolk or albumen of developing eggs, perhaps as a conse-

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quence of colonization of different regions of the reproductive tract (Humphrey et al., 1989, 1991; Gast and Beard, 1990; Bichler et al., 1996; Gast and Holt, 2000b). Detailed examination of eggs from experimentally infected hens has suggested that *S. enteritidis* is deposited more often in association with the yolk (vitelline) membrane than in the interior contents of the yolk (Gast and Holt, 2001c). The low frequency at which contaminated eggs have been found to contain large numbers of *S. enteritidis* cells provides additional (albeit indirect) evidence that initial bacterial deposition inside the nutrient-rich yolk may be a uncommon event.

Even if the initial site of *Salmonella* deposition is the exterior surface of the vitelline membrane or in adjacent regions of the albumen, penetration of the membrane could lead to extensive multiplication inside the yolk. Penetration of *S. enteritidis* through the vitelline membrane has been reported (at a wide range of frequencies) using several types of in vitro contamination models (Hammack et al., 1993; Humphrey and Whitehead, 1993; Braun and Fehlhaber, 1995; Gast and Holt, 2000a, 2001c), although no penetration was observed on other occasions (Fleischman et al., 2003). Because most experiments involving in vitro bacterial penetration through the yolk membrane have been conducted with single strains of *S. enteritidis*, the actual extent to which this ability is distributed among other strains of *S. enteritidis* or among strains of other serotypes such as *S. heidelberg* is not known. The objectives of the present study were to determine the frequencies at which a strain of *S. enteritidis* and 2 strains of *S. heidelberg* were able to penetrate the vitelline membrane to reach the yolk contents in an in vitro contamination model, and to determine whether variants of these strains obtained by re-isolation from eggs laid by experimentally infected hens differed from the parent strains in their abilities to penetrate the yolk membrane.

MATERIALS AND METHODS

Preparation of S. enteritidis and S. heidelberg Cultures

Frozen *Salmonella* cultures were resuscitated by transfer into tryptone soy broth[2] for 2 successive 24-h cycles of incubation at 37°C. After the cell densities of the incubated cultures were estimated by determining their optical densities at 600 nm, further dilution in saline produced the desired final cell concentration (confirmed by subsequent plate counts). Six *Salmonella* cultures were used: 3 "parent" strains and 3 "passaged" strains. One parent strain was a phage type 13a *S. enteritidis* isolate (designated strain 6) that has been consistently associated with egg contamination in experimental infection studies (Gast and Holt, 2000b, 2001b, 2001c; Gast et al., 2003). The other 2 parent strains were *S. heidelberg* isolates (designated strains 4 and 11), provided by B. Swaminathan[3] and originally obtained from infected humans during disease outbreaks for which eggs were an implicated food source. The 3 passaged *Salmonella* strains (designated *S. enteritidis* 6p, *S. heidelberg* 4p, and *S. heidelberg* 11p) were isolated from eggs laid by hens that were infected with the parent strains in a prior study (Gast et al., 2004).

Preparation, Inoculation, and Incubation of Egg Contents Samples

Freshly collected eggs from our laboratory's specific-pathogen-free flock of Single Comb White Leghorn chickens were aseptically broken, their contents (yolk and albumen) were separated, and each yolk was transferred into the bottom of a sterile 50-mL plastic centrifuge tube. Each yolk was then inoculated with *Salmonella* by using a pipette to dispense 0.1 mL of the appropriate broth culture (containing approximately 100 cfu) onto the exterior surface of the vitelline membrane. After the inoculated yolk samples were held for 5 min at room temperature (approximately 24°C), the albumen from a single egg was gently poured into each tube. Twenty egg contents samples were inoculated with each of the 6 *Salmonella* strains, and 6 uninoculated samples were retained as negative controls. After preparation, all samples were incubated for 24 h at 30°C.

Enumeration of S. enteritidis and S. heidelberg inside Egg Yolks After Incubation

Each incubated egg contents sample was poured into a sterile plastic Petri dish. A small area of the yolk membrane was seared with a flame-heated steel spatula to destroy surface bacteria present in that region. A sterile syringe was then inserted through the seared area of the membrane to remove 5 mL of interior yolk contents (free of membrane material). The concentration of *S. enteritidis* or *S. heidelberg* in the yolk contents was determined by making 10-fold dilutions of each sample in 0.85% saline and spreading aliquots of each dilution onto plates of brilliant green agar[4] supplemented with 0.02 mg/mL of novobiocin.[5] The agar plates were incubated for 24 h at 37°C and typical *Salmonella* colonies were identified (Waltman et al., 1998) and counted. The detection threshold of this procedure was 10 cfu/mL.

Statistical Analysis

Significant differences ($P < 0.05$) between treatment groups in the frequency of isolation and mean concentration of *Salmonella* strains in the interior contents of yolk samples after incubation were determined by

[2]Oxoid Ltd., Basingstoke, UK.
[3]Centers for Disease Control and Prevention, Atlanta, GA.
[4]Becton-Dickinson Co., Sparks, MD.
[5]Sigma Chemical Co., St. Louis, MO.

TABLE 1. Isolation and enumeration of *Salmonella enteritidis* and *Salmonella heidelberg* strains in the contents of egg yolk samples[1]

Strain[2]	*Salmonella*-positive yolk samples/total	*Salmonella* concentration in yolk samples ($\log_{10}$ cfu/mL)
Salmonella enteritidis 6	$5/20^{ab}$	0.633^{ab}
S. enteritidis 6p	$11/20^{cd}$	1.723^{cd}
Salmonella heidelberg 4	$2/20^{a}$	0.229^{a}
S. heidelberg 4p	$7/20^{bc}$	1.252^{bc}
S. heidelberg 11	$3/20^{ab}$	0.406^{ab}
S. heidelberg 11p	$13/20^{d}$	2.574^{d}

[a–d]Values in columns that share no common superscripts differ significantly ($P < 0.05$).

[1]After inoculation with approximately 10^2 cfu onto the vitelline membrane and incubation at 30°C for 24 h.

[2]Strains designated with a "p" were obtained by oral inoculation of laying hens with the corresponding parent strain and subsequent reisolation from egg contents.

applying the Mann-Whitney test. Data were analyzed using Instat biostatistics software.[6]

RESULTS AND DISCUSSION

After inoculation onto the exterior surfaces of egg yolk vitelline membranes, all 6 *S. enteritidis* and *S. heidelberg* strains were able to penetrate into the interior yolk contents during 24 h of incubation at 30°C (Table 1). The frequencies of recovery of the 3 parent strains (*S. enteritidis* 6, *S. heidelberg* 4, and *S. heidelberg* 11) from yolk contents did not differ significantly, ranging from 10 to 25%. The mean $\log_{10}$ concentrations of the 3 parent strains found in yolk contents were not significantly different from each other, ranging from 0.229 to 0.633 cfu/mL. Both the frequencies of isolation and the $\log_{10}$ concentrations in egg yolk contents were significantly greater ($P < 0.05$) for all 3 strains obtained by passage through chickens (*S. enteritidis* 6p, *S. heidelberg* 4p, and *S. heidelberg* 11p) than for the corresponding parent strains. The observed frequencies of penetration of the passaged strains into the interiors of egg yolks ranged from 35 to 65% and the mean $\log_{10}$ concentrations of these strains in yolk contents ranged from 1.252 to 2.574 cfu/mL. None of the uninoculated negative control samples was found to be *Salmonella*-positive after incubation.

Rapid refrigeration of freshly laid eggs restricts the multiplication of bacterial pathogens (Chen et al., 2002) and can thereby minimize the risk of disease transmission to consumers. Previous investigations have shown that infected hens may deposit *S. enteritidis* either in the albumen or on the vitelline membrane of eggs, but the pathogen is rarely deposited inside the nutrient-rich contents of the yolk (Gast and Holt, 2001c; Gast et al., 2003). However, penetration of contaminants through the vitelline membrane could provide an opportunity for extensive bacterial multiplication inside the yolk before egg refrigeration achieves growth-inhibiting internal temperatures. Penetration of *S. enteritidis* through the yolk membrane has been reported in several previous experiments based on in vitro egg contamination models (Hammack et al., 1993; Humphrey and Whitehead, 1993; Braun and Fehlhaber, 1995; Gast and Holt, 2000a, 2001c). In the present study, entry into the yolk interior and a modest degree of multiplication from the initial inoculum levels were seen for strains of *S. enteritidis* and *S. heidelberg*. The observed multiplication of *Salmonella* could have occurred before or after bacterial arrival inside yolks, but the relatively small numbers of *Salmonella* cells detected in the nutritionally abundant yolk contents suggests that penetration likely proceeded rather slowly or happened very late in the incubation period. The 3 *Salmonella* parent strains were isolated from yolk contents at relatively low frequencies, but the isolates derived by in vivo passage through chickens penetrated into yolks significantly more often. In an earlier study, several rounds of passage of an *S. enteritidis* strain through laying hens increased its ability to cause egg contamination (Gast et al., 2003).

Using an in vitro model for egg contamination that was somewhat similar to the approach used in the present study, Fleischman et al. (2003) reported substantial multiplication of *S. enteritidis* on the vitelline membrane during incubation at 27 to 37°C, but no penetration into the yolk interior was detected. In this earlier experiment, only 1 mL of yolk contents was sampled for *S. enteritidis* after incubation, whereas the 5-mL sample of yolk contents that was removed for testing in the present study may have increased the probability of finding small numbers of *Salmonella* cells. Another issue that could affect the results in these types of experiments concerns the selection of *Salmonella* strains for inoculation into eggs. Fleischman et al. (2003) used a standard reference strain of *S. enteritidis*, whereas the present study employed isolates from eggs or egg-associated disease outbreaks. Prior research established that *S. enteritidis* strains could differ significantly in their growth properties in eggs (Gast and Holt, 2001a; Cogan et al., 2004). Moreover, isolates from eggs have been found to differ from isolates obtained from other poultry sources in the composition of their surface lipopolysaccharides (Guard-Bouldin et al., 2004).

The degree of correlation between in vitro contamination models and naturally contaminated eggs remains somewhat uncertain. The small numbers of *S. enteritidis* cells that have typically been reported inside freshly laid, naturally contaminated eggs (Humphrey et al., 1991) may indicate that penetration into the growth-supporting yolk contents does not normally occur at a high frequency. Entry into the yolk could become more likely over time as albumen viscosity and vitelline membrane integrity decline, especially at elevated temperatures (Humphrey and Whitehead, 1993; Hara-Kudo et al., 2001; Latimer et al., 2002; Messens et al., 2004). The in vitro contamination model used in the present study might accelerate the natural bacterial penetration pro-

[6]GraphPad Software, San Diego, CA.

cess by maintaining a high incubation temperature. In addition, the inoculation of an enrichment broth culture directly onto the vitelline membrane may alter the interaction between bacteria and membrane compared with naturally occurring deposition of *Salmonella* in eggs. Nevertheless, the present study suggests that *S. enteritidis* and *S. heidelberg* deposited outside the vitelline membrane of freshly laid eggs can sometimes reach the yolk contents and begin to multiply within a single day of storage at a warm temperature, although this penetration does not appear to progress rapidly. Moreover, in vivo passage into eggs laid by infected chickens can increase the ability of *Salmonella* strains to penetrate into yolks.

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